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BHC80 is Critical in Suppression of Snail-LSD1 Interaction and Breast Cancer Metastasis

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14. ABSTRACT

By using a tandem affinity-mass spectrometry coupled analysis, we identified Snail-interacting proteins including LSD1, BHC80 and PARP1. Based on our preliminary data, Snail uses its highly conserved SNAG domain to recruit LSD1 to its target gene promoter for transcription repression. Among the Snail-interacting proteins, PARP1 is critical in regulating the protein stability of Snail and LSD1, as well as Snail/LSD1 binding to the target gene promoter area. According to the modified Statement of Work, we focused on clarifying the mechanism of PARP1-mediated cancer development. We demonstrated that Snail cooperates with LSD1 to repress PTEN in a PARP1-dependent manner. Upon doxorubicin treatment, Snail becomes tightly associated with PARP1 through its pADPr-binding motif and is subject to poly(ADP-ribosyl)ation. This modification can enhance Snail-LSD1 interaction and promote the recruitment of LSD1 to PTEN promoter, where LSD1 removes methylation on histone H3 lysine 4 for transcription repression. Furthermore, treatment of tumor cells with PARP1 inhibitor AZD2281 can compromise doxorubicin-induced PTEN suppression and enhance the inhibitory effect of doxorubicin. Together, we proposed a tentative drug-resistant mechanism through which tumor cells defend themselves against DNA damage-induced apoptosis. PARP1 inhibitors in combination with DNA damaging reagents might represent a promising treatment strategy targeting tumors with over-activated Snail and LSD1. We submitted the manuscript to Cell Cycle, and the paper was accepted for publication in March 2014. We are currently continuing to characterize other Snail-interacting proteins to get a clearer picture of Snail-mediated cancer progression.

15. SUBJECT TERMS
Snail, PARP1, LSD1, cancer

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Introduction

Cancer cells distinguish themselves from their normal siblings with the capability of evading apoptosis and presenting uncontrolled cell division, along with acquiring malignant characteristics such as invasion and metastasis. The conventional chemotherapeutic drugs function by introducing DNA damage to impair cell division. Since most cancer cells outgrow their normal counterparts, the property of rapid DNA-replication makes them more vulnerable to the DNA lesions. On the other side, a portion of cancer cells have their own defensive strategies, either harboring intrinsic capability to escape apoptosis or developing resistance following drug exposure, which allow for tumor recurrence and progression. Chemoresistance is determined by aberrant genetic settings in combination with diverse epigenetic alterations, reflected by abnormal signaling pathways controlling drug accumulation and distribution, cell proliferation, DNA repair and apoptosis. While great effort has been made to elucidate the underlying mechanism, our knowledge on drug resistance is still fragmentary.

As a typical oncogene, the zinc finger transcription factor Snail is over-expressed in various types of tumors. Snail functions not only as a master regulator of epithelial-mesenchymal transition (EMT) that promotes tumor metastasis, but also as an important molecule that induces immunosuppression, bestows cancer cells with stem-like traits, and mediates cancer cell survival. In terms of cell survival, Snail expression has been demonstrated to confer chemoresistance on breast, colon, lung and pancreatic cancer cells. Mechanistically, Snail can become stabilized and bind to PTEN promoter to repress its transcription during radiation-induced apoptosis. It has also been documented that upon doxorubicin treatment, the pro-survival Akt pathway becomes activated to render breast cancer cells resistant to drug-induced apoptosis. Based on the findings that PTEN negatively regulates the PI3K/Akt pathway, and that over-expression of Akt can induce NF-κB-dependent Snail activation, there is a plausible positive feedback loop in which Snail boosts its own transcription through PTEN suppression.

To obtain a clearer picture of Snail-mediated tumor survival and progression, we recently applied an affinity purification-mass spectrometry coupled analysis to identify Snail-interacting proteins, among which are lysine-specific demethylase 1 (LSD1) and poly(ADP-ribose) polymerase 1 (PARP1). As the first identified histone demethylase, LSD1 specifically removes methylation on histone H3 lysine 4 (H3K4me), which is a transcription activation mark. LSD1 plays an essential role during development, and over-expression of LSD1 has been correlated with malignant progression of multiple cancers, including primary neuroblastic tumors, prostate cancer, and ER-negative breast cancer. In our recent study, we demonstrated that Snail uses its SNAG domain as a pseudo-substrate to recruit LSD1 to its target gene E-cadherin promoter for transcription suppression and EMT induction. Furthermore, we found that the expression of Snail was significantly co-related with that of LSD1 in multiple human breast cancer tissues. Interestingly, according to other recent studies, LSD1 can either render tumor cells resistant to DNA damage or reversely prompt cells to undergo apoptosis in different biological settings, indicating that LSD1 plays a role in cell survival. As mentioned, one of the critical oncogenic roles of Snail lies in apoptosis protection notably through transcriptional repression of PTEN, which serves as a negative regulator of Akt signaling. It would be interesting to find out if LSD1 is involved in Snail-mediated PTEN suppression and cell survival.

Besides LSD1, PARP1 is another intriguing candidate that serves as a key factor in DNA repair and cell survival. PARP1 becomes immediately activated in response to single-strand DNA breaks, and utilizes NAD^+ as substrate to synthesize poly(ADP-ribose) polymer (pADPr), which functions as a signal for recruiting other DNA-repairing enzymes. If not repaired, single-strand DNA breaks will cause the replication fork to stall and double-strand DNA breaks to accumulate during DNA replication. Since some breast cancers have defects in BRCA1/BRCA2-mediated homologous recombination (HR) repair pathway that deals with double-strand breaks, they would rely on PARP1 to repair DNA lesions. These cancer cells are hypothesized to be highly sensitive to PARP inhibitors under various cellular stresses. Indeed, PARP inhibitors have shown more toxicity in cancer cell lines as well as human tumors with BRCA1/BRCA2 deficiency. In addition to DNA repair, PARP1 is actively involved in transcription regulation, either through directly modulating chromatin structure or by regulating other chromatin-modifying enzymes and transcription factors.

In the current study, we demonstrated that doxorubicin treatment can enhance Snail-LSD1 interaction in a PARP1-dependent manner. In addition, Snail contains a potential pADPr-binding motif and is subject to poly(ADP-ribosyl)ation. Our data also suggested that the enzymatic activity of PARP1 is required for Snail-LSD1 binding to the PTEN promoter; upon binding, LSD1 demethylates histone H3 lysine 4 at the promoter region in favor of PTEN transcription suppression and up-regulation of Akt phosphorylation. Furthermore, we found that PARP1 inhibitor AZD2281 can enhance the killing effect of doxorubicin on selective breast and colon cancer cells. Together, we proposed a new mechanism adopted by cancer cells to defend themselves against DNA damage-induced apoptosis, which gives us new implications on the design of efficient cancer treatment strategies.

Body

According to the renewed Statement of Work (SOW) as listed below, we focus on the regulative role of PARP1 in Snail/LSD1 complex. Based on our research results, we submitted a manuscript to Cell Cycle, and our paper was accepted for publication (Ref. 1). We attached the article as substitution for part of our research accomplishments.

SOW – Study 1: How does PARP1 potentially regulate Snail/LSD1 complex?

1a To confirm the physical interaction of PARP1 and Snail

Please refer to Figure 1 and related paragraphs of the attached article to be published in Cell Cycle.

1b Can PARP1 mediate Snail-LSD1 interaction?

Please refer to Figure 2 and related paragraphs of the attached article to be published in Cell Cycle.

1c To identify the specific mechanism of how PAPR1 mediate Snail-LSD1 interaction

Please refer to Figure 3 and related paragraphs of the attached article to be published in Cell Cycle.

SOW – Study 2: Does PARP1 mediated Snail-LSD1 interaction have any biological significance?

2a Can PARP1 mediate Snail/LSD1 binding to PTEN promoter?

Please refer to Figure 4 and related paragraphs of the attached article to be published in Cell Cycle.

2b To identify the biological function of Snail/LSD1/PARP1 complex

Please refer to Figure 5 and related paragraphs of the attached article to be published in Cell Cycle.

2c Manuscript preparation and submission

The manuscript has been accepted for publication in Cell Cycle.

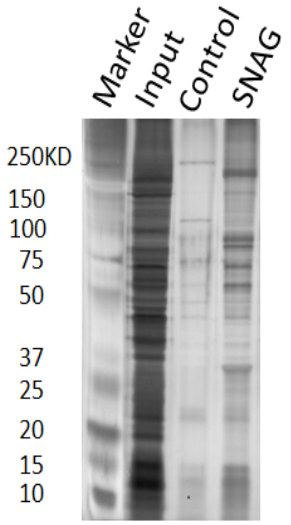
SOW – Study 3: Functional characterization of other Snail-interacting proteins

3a Identification of SNAG-interacting proteins

To further identify SNAG-associated proteins besides LSD1, we applied peptide pulldown-mass spectrometry-coupled analysis. The protein complex was separated by SDS-PAGE and subjected to silver staining as shown in Figure 1. The protein bands were then sent for Mass Spectrometry analysis. The protein identified are LSD1, CoREST, BHC80, HDAC1/2, EZH2, KDM5B (lysine (K)-specific demethylase 5B, which is an H3K4me3-specific demethylase) and NSD2 (Nuclear receptor-binding SET domain protein 2, which harbors histone lysine methyltransferases activity), among others.

Figure 1 *Identification of SNAG peptide-interacting proteins.*

Peptide pulldown samples were separated on SDS-PAGE and subjected to silver staining before mass-spectrometry analysis. Peptide-absent sample was used as negative control.



3b Characterization of SNAG-interacting proteins

We carefully searched literatures on the newly identified candidates and picked a couple of interesting candidates for our further research. We are currently working on the role of NSD2 and KDM5B in Snail-mediated tumor metastasis. We have confirmed the physical interactions of Snail with NSD2 and KDM5B through co-immunoprecipitation. The study on the biological functions of these proteins is currently underway.

3c Manuscript preparation and submission

We are currently collecting data for manuscript preparation and future publications.

Key research accomplishments

In summary, we demonstrated that PARP1-mediated poly(ADP-ribosyl)ation of Snail is critical for Snail-LSD1 complex formation and the downstream PTEN suppression. Our study not only provides a new insight into the working mechanism of the Snail transcriptional machinery, but also explores the potential application of PARP inhibitors in conjunction with DNA damage-inducing reagents in targeting cancer cells. As PARP inhibitors are thrust into the limelight by the encouraging results of early clinical trials, our study would provide extra impetus for future drug development and help to diversify cancer treatment strategies.

In addition, through application of SNAG-peptide pulldown assay, we identified several interesting SNAG-interacting proteins. Functional characterization of these proteins will hopefully provide us with a clearer picture of Snail-mediated cancer progression.

Reportable outcomes

Publications:

The manuscript entitled "Doxorubicin enhances Snail/LSD1-mediated PTEN suppression in a PARP1-dependent manner" was accepted for publication in Cell Cycle in March, 2014 (Ref. 47).

Based on our recent results, a review entitled "Epigenetic regulation of EMT: the Snail story" was prepared and published in Current Pharmaceutical Design in July, 2013 (Ref. 48).

We attached both articles in the appendix part.

Degree earning: Ph.D. degree was obtained in December of 2012.

Conclusion

In this study, we demonstrated that through interacting with and poly(ADP-ribosyl)ating Snail, PARP promotes the formation of Snail-LSD1 repressive complex and enhances the binding of the complex at the PTEN promoter for transcription suppression. Previously we have shown that Snail uses its highly conserved SNAG domain as a histone mimicking "hook" to recruit LSD1 to its target gene promoters. The involvement of PARP1 in this regulation process particularly under the condition of DNA damage adds another layer to this delicate transcriptional machinery. As the founding member of the PARP superfamily,

PARP1 is a multifunctional protein that not only plays a role in DNA repair, but also participates in gene transcription regulation. The effect of PARP1 could either be stimulatory or inhibitory, depending on the specific environmental context and cellular signals. In the very case discussed here, PAPR1 provides a stimulatory effect on the Snail-LSD1 complex under DNA damage. Upon activation by doxorubicin, PARP1 uses its pADPr for association with the pADPr-binding motif of Snail, and furthermore promotes the interaction of Snail with LSD1. Disruption of the pADPr-binding motif by point mutation not only resulted in loss of Snail-PARP1 association, but also strikingly compromised Snail-LSD1 complex interaction. In addition, we found that Snail could undergo poly(ADP-ribosyl)ation on DNA damage condition. Based on these results, together with previous finding that Snail interacts with LSD1 through its SNAG domain, we reasoned that binding and modification of Snail by PARP1 could change the conformation of Snail and potentially expose its LSD1-binding motif on the SNAG domain to facilitate Snail-LSD1 interaction. Therefore, LSD1 can be recruited by Snail to the target gene (PTEN in this case) promoter, where it demethylates histone H3 lysine 4 in favor of transcription repression. The model provided here illustrated a tantalizing mechanism of how cancer cells defense themselves against DNA damage and try to evade PTEN-mediated apoptosis. A detailed computer-based structure analysis would hopefully further illustrate this dynamic regulatory process and will be done in the near future. We also tried to explore our findings by specifying the residues on Snail protein that are subject to poly(ADP-ribosyl)ation. Mutation of the lysine residue on the pADPr-binding motif of Snail did not significantly compromise the level of poly(ADP-ribosyl)ation, neither did mutations on Lys9, Asp12 or Lys16 of SNAG domain (data not shown), indicating that Snail can undergo poly(ADP-ribosyl)ation on multiple residues, which remain to be defined in the future. Together, our study illustrated the cooperation of Snail, LSD1 and PARP1 in PTEN transcription suppression under DNA damage condition. Previous to our study, there have been reports demonstrating the dynamic roles of PARP1 in gene transcription regulation. For example, upon binding to nucleosomes, PARP1 may regulate the compaction/decompaction of chromatin; PARP1 may also exclude histone H1 from its target gene promoters in favor of transcription activation[37]. In addition, PARP1 has been shown to interact noncovalently with DNA methyltransferase 1 (Dnmt1) and inhibit its enzymatic activity, in such way that it regulates genomic methylation patterns. As another typical example, PARP1 can dissociate Smad complexes from DNA through poly(ADP-ribosyl)ating Smad3 and Smad4, therefore attenuating Smad-mediated transcription and inhibiting TGF-β-induced epithelial-mesenchymal transition (EMT). Since we have demonstrated before the function of Snail-LSD1 on E-cadherin suppression and EMT induction, there is also a possibility that PARP1 participates in this regulation process. In contrast to the Smad-PARP1 model, however, poly(ADP-ribosyl)ation of Snail by PARP1 would rather enhance the inhibitory effect of Snail-LSD1 on E-cadherin and promote EMT. We reason that in different biological settings, cells may rely on different transcriptional machineries to turn on or off the expression of specific genes in response to various signals, or for the adaptation of different extracellular and intracellular stresses. Our hypothesis is further supported by recent finding that PARP1 can poly(ADP-ribosyl)ate and stabilize Snail and promote Snail-mediated EMT. We speculate that PARP1 regulates Snail-mediated EMT using a more complicated and delicate mechanism, probably through promoting the recruitment or release of other histone-modifying enzymes and cofactors (LSD1 for example) to further modify chromatin. Since both E-cadherin and PTEN loss can promote the generation of cancer stem cells, another intriguing question is to what extent the Snail-LSD1-PARP1 complex contributes to cell stemness. While more work is still needed for clarification of the whole mechanism, our current study provides important information for the completion of the model of Snail-mediated transcription regulation.

The second insight provided by our study lies in the finding that PARP inhibitors in conjunction with DNA-damaging reagents may represent an effective treatment strategy against a much wider range of cancers. While the conventional chemotherapeutic drugs such as doxorubicin function by targeting DNA synthesis and cell division, unfortunately they are not smart in pinpointing cancer cells; rather they also do harm to normal cells with rapid dividing property. Even worse, many solid tumors continually undergoing chemotherapy will ultimately acquire drug resistance. Many cancer cells have defective DNA repair pathways. For example, BRCA1/2 mutations are found in breast and ovarian cancers, and mutations of ataxia telangiectasia mutated (ATM) gene are identified in lymphoid malignancies. In this regard, targeting DNA repair machineries is a promising strategy for achieving synthetic lethality on cancer treatments.

Indeed, PARP inhibitors are prevailingly considered as treatment options against BRCA1/2-deficient tumors due to the synthetic lethality effect. Under the same rationale, PARP1 inhibitors are also used in the treatment of tumors deficient in PTEN, which plays a critical role in the expression of the repair protein RAD51. Currently, different PARP inhibitors combined with DNA-damaging reagents are under investigation in several clinical trials. We have shown the enhanced suppressive effect of doxorubicin-AZD2281 combination on BRCA1/2 and PTEN wild-type MDA-MB-157 and HCT116 cells. Based on our results, we argue that in addition to the induction of DNA damage, doxorubicin treatment also enhances Snail-LSD1 mediated PTEN suppression in a PARP1-dependent manner, which results in phosphorylation and activation of pro-survival Akt. Inhibition of PARP1 can compromise this undesirable effect while synergizing the DNA-damaging effect of doxorubicin to efficiently suppress tumor cells. While *in vivo* experiments are required to consolidate our results as well as to evaluate the long-term effect of PARP1 inhibition, our data expands potential therapeutic benefits of PARP1 inhibitors, especially on tumors with high levels of Snail and LSD1 expression. Furthermore, it is inviting to see if PARP1 inhibitors can synergize with LSD1 inhibitors and novel SNAG domain-mimicking compounds that block Snail-LSD1 interaction to treat these kinds of cancers.

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Doxorubicin Enhances Snail/LSD1-mediated PTEN Suppression in a PARP1-dependent Manner

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Running title

Doxorubicin enhances Snail-LSD1 interaction via poly(ADP-ribosyl)ation

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ABSTRACT

The transcription factor Snail not only functions as a master regulator of epithelial-mesenchymal transition (EMT), but also mediates cell proliferation and survival. While previous studies have showed that Snail protects tumor cells from apoptosis through transcriptional repression of PTEN, the specific mechanism remains unclear. In this study, we demonstrated that Snail cooperates with LSD1 to repress PTEN in a PARP1-dependent manner. Upon doxorubicin treatment, Snail becomes tightly associated with PARP1 through its pADPr-binding motif and is subject to poly(ADP-ribosyl)ation. This modification can enhance Snail-LSD1 interaction and promote the recruitment of LSD1 to PTEN promoter, where LSD1 removes methylation on histone H3 lysine 4 for transcription repression. Furthermore, treatment of tumor cells with PARP1 inhibitor AZD2281 can compromise doxorubicin-induced PTEN suppression and enhance the inhibitory effect of doxorubicin. Together, we proposed a tentative drug-resistant mechanism through which tumor cells defend themselves against DNA damage-induced apoptosis. PARP1 inhibitors in combination with DNA damaging reagents might represent a promising treatment strategy targeting tumors with over-activated Snail and LSD1.

INTRODUCTION

Cancer cells distinguish themselves from their normal siblings with the capability of evading apoptosis and presenting uncontrolled cell division, along with acquiring malignant characteristics such as invasion and metastasis. The conventional chemotherapeutic drugs function by introducing DNA damage to impair cell division. Since most cancer cells outgrow their normal counterparts, the property of rapid DNA-replication makes them more vulnerable to the DNA lesions. On the other side, a portion of cancer cells have their own defensive strategies, either harboring intrinsic capability to escape apoptosis or developing resistance following drug exposure, which allow for tumor recurrence and progression. Chemoresistance is determined by aberrant genetic settings in combination with diverse epigenetic alterations, reflected by abnormal signaling pathways controlling drug accumulation and distribution, cell proliferation, DNA repair and apoptosis[1]. While great effort has been made to elucidate the underlying mechanism, our knowledge on drug resistance is still fragmentary.

As a typical oncogene, the zinc finger transcription factor Snail is over-expressed in various types of tumors[2, 3]. Snail functions not only as a master regulator of epithelial-mesenchymal transition (EMT) that promotes tumor metastasis[4-8], but also as an important molecule that induces immunosuppression, bestows cancer cells with stem-like traits, and mediates cancer cell survival[9]. In terms of cell survival, Snail expression has been demonstrated to confer chemoresistance on breast, colon, lung and pancreatic cancer cells[10-13]. Mechanistically, Snail can become stabilized and bind to PTEN promoter to repress its transcription during γ radiation-induced apoptosis[14]. It has also been documented that upon doxorubicin treatment, the pro-survival Akt pathway becomes activated to render breast cancer cells resistant to drug-induced apoptosis[15]. Based on the findings that PTEN negatively regulates the PI3K/Akt pathway[16], and that overexpression of Akt can induce NF-κB-dependent Snail activation[17], there is a plausible positive feedback loop in which Snail boosts its own transcription through PTEN suppression.

To obtain a clearer picture of Snail-mediated tumor survival and progression, we recently applied an affinity purification-mass spectrometry coupled analysis to identify Snail-interacting proteins, among which are lysine-specific demethylase 1 (LSD1) and poly(ADP-ribose) polymerase 1 (PARP1)[18]. As the first identified histone demethylase, LSD1 specifically removes methylation on histone H3 lysine 4 (H3K4me), which is a transcription activation mark[19]. LSD1 plays an essential role during development, and overexpression of LSD1 has been correlated with malignant progression of multiple cancers, including primary neuroblastic tumors, prostate cancer, and ER-negative breast cancer[20-22]. In our recent study, we demonstrated that Snail uses its SNAG domain as a pseudo-substrate to recruit LSD1 to its target gene E-cadherin promoter for transcription suppression and EMT induction[18]. Furthermore, we found that the expression of Snail was significantly co-related with that of LSD1 in multiple human breast cancer tissues[18].

Interestingly, according to other recent studies, LSD1 can either render tumor cells resistant to DNA damage or reversely prompt cells to undergo apoptosis in different biological settings, indicating that LSD1 plays a role in cell survival[23-26]. As mentioned, one of the critical oncogenic roles of Snail lies in apoptosis protection notably through transcriptional repression of PTEN, which serves as a negative regulator of Akt signaling. It would be interesting to find out if LSD1 is involved in Snail-mediated PTEN suppression and cell survival.

Besides LSD1, PARP1 is another intriguing candidate that serves as a key factor in DNA repair and cell survival. PARP1 becomes immediately activated in response to single-strand DNA breaks, and utilizes NAD^+ as substrate to synthesize poly(ADP-ribose) polymer (pADPr), which functions as a signal for recruiting other DNA-repairing enzymes[27, 28]. If not repaired, single-strand DNA breaks will cause the replication fork to stall and double-strand DNA breaks to accumulate during DNA replication[29]. Since some breast cancers have defects in BRCA1/BRCA2-mediated homologous recombination (HR) repair pathway that deals with double-strand breaks, they would rely on PARP1 to repair DNA lesions. These cancer cells are hypothesized to be highly sensitive to PARP inhibitors under various cellular stresses. Indeed, PARP inhibitors have shown more toxicity in cancer cell lines as well as human tumors with BRCA1/BRCA2 deficiency[30-32]. In addition to DNA repair, PARP1 is actively involved in transcription regulation, either through directly modulating chromatin structure or by regulating other chromatin-modifying enzymes and transcription factors[33, 34].

In the current study, we extended our findings by demonstrating that doxorubicin treatment can enhance Snail-LSD1 interaction in a PARP1-dependent manner. In addition, Snail contains a potential pADPr-binding motif and is subject to poly(ADP-ribosyl)ation. Our data also suggested that the enzymatic activity of PARP1 is required for Snail-LSD1 binding to the PTEN promoter; upon binding, LSD1 demethylates histone H3 lysine 4 at the promoter region in favor of PTEN transcription suppression and upregulation of Akt phosphorylation. Furthermore, we found that PARP1 inhibitor AZD2281 can enhance the killing effect of doxorubicin on selective breast and colon cancer cells. Together, we proposed a new mechanism adopted by cancer cells to defend themselves against DNA damage-induced apoptosis, which gives us new implications on the design of efficient cancer treatment strategies.

RESULTS

PARP1-Snail interaction is enhanced by doxorubicin treatment

We have previously established a stable HEK293 cell line expressing dual-tagged Snail[18]. To identify Snail-interacting proteins, a two-step, sequential protein purification was performed to isolate Snail complexes, which were subjected to mass spectrometry analysis. PARP1, which has a molecular weight of around 116kDa, was identified in the complexes besides several already known Snail partners such as GSK3-β, β-Trcp, PRMT5 and LSD1 (**Figure 1A**). The roles of PARP1 in DNA damage repair as well as transcription regulation make it a promising candidate for investigating the mechanism of Snail-mediated PTEN suppression and cell survival. To confirm the physical interaction of Snail-PARP1, we performed co-immunoprecipitation experiments using HEK293 cells over-expressing Snail-HA and Flag-PARP1, as well as breast cancer cell line MDA-MB-157 and colon cancer cell line HCT116. As shown in Figure 1B and 1C, Snail and PARP1 proteins showed relatively modest interaction in all of the three cell lines. Interestingly, the protein interaction was significantly enhanced when the cells were treated with doxorubicin, indicating that upon DNA damage, PARP1 becomes tightly associated with Snail.

PARP1 positively regulates Snail-LSD1 interaction through poly(ADP-ribosyl)ation of Snail

Since we have recently demonstrated that Snail and LSD1 can form a repressor complex, we examined if PARP1 can facilitate the association of Snail with LSD1. In HEK293 cells overexpressing Snail and LSD1, doxorubicin treatment significantly enhanced Snail-LSD1 binding, and consistent results could be obtained by overexpressing PARP1 (**Figure 2A**). In MDA-MB-157 and HCT116 cells, while doxorubicin consistently enhanced Snail-LSD1 binding, either PARP1 knockdown or treatment of PARP1 inhibitor AZD2281 significantly reduced Snail-LSD1 association (**Figure 2B**). These results indicated that PARP1 promotes the formation of the Snail-LSD1 complex.

Through sequence alignment we identified three highly conserved residues Arg151, Lys152 and Ala153 of Snail protein to be in concert with the corresponding residues of the previously established pADPr binding motif, in which the positively charged lysine and arginine are strictly followed by one small non-polar amine acid (alanine, isoleucine, leucine or valine) (**Figure 3A**). Considering that PARP1 became activated and tightly bound to Snail upon DNA damage, we went on to investigate whether Snail can interact with PARP1 through its potential pADPr-binding motif. First we generated Snail point mutant R151A/K152A and examined its interaction with PARP1. As shown in Figure 3B, the mutant Snail significantly lost PARP1 binding affinity compared to the wild-type protein, indicating that R151/K152 of Snail are critical for PARP1 association. Interestingly, the mutant also significantly decreased the binding affinity for LSD1, further confirming that the presence of PARP1 is required for Snail-LSD1 association

(**Figure 3C**). Consistently, when the cells were treated with gallotannin, an inhibitor of poly(ADP-ribose) glycohydrolase (PARG) which catalyzes the degradation of pADPr (an inverse step of poly-ADP-ribosylation), the association of Snail-LSD1 was significantly enhanced (**Figure 3D**). Furthermore, the Snail mutant became less stable compared to the wild-type protein (**Figure 3E**), which was in accord with our previous finding that formation of Snail-LSD1 complex was required for maintaining the stability of each protein component[18].

Upon activation, PARP1 functions by attaching pADPr chain on specific glutamate, aspartate or lysine residues of its target proteins. To investigate whether Snail can undergo poly(ADP-ribosyl)ation upon association with PARP1, we immunoprecipitated Snail protein from the abovementioned stable HEK293 cells, and performed Western-blot using antibody against pADPr. As shown in Figure 3F, Snail protein was poly(ADP-ribosyl)ated, the effect of which could be enhanced by doxorubicin and suppressed by AZD2281. Together, we demonstrated that 1) PARP1 enhances Snail-LSD1 association as well as their protein stability through interacting with a potential pADPr-binding motif of Snail; and 2) Snail protein is subject to PARP1-mediated poly(ADP-ribosyl)ation.

The enzymatic activity of PARP1 is required for Snail-LSD1 binding to PTEN promoter

Previous studies have demonstrated that Snail can bind to PTEN promoter to repress its transcription. The formation of Snail-LSD1-PARP1 complex under DNA damage condition prompted us to investigate how this protein complex potentially cooperates to downregulate PTEN in favor of tumor cell survival. Since Snail interacts with LSD1 through its SNAG domain, we reasoned that Snail can recruit LSD1 to PTEN promoter for H3K4 demethylation and gene suppression. We then performed chromatin immunoprecipitation (ChIP) assays to test this hypothesis. Indeed, both Snail and LSD1 could interact with PTEN promoter in MDA-MB-157 and HCT116 cells (**Figure 4A**). Interestingly, the promoter-binding of both proteins was significantly enhanced upon doxorubicin treatment, indicating that PARP1 becomes activated in response to DNA-damaging reagent and promotes the association of Snail/LSD1 at the PTEN promoter. Also as expected, AZD2281 treatment or PARP1-knockdown inhibited the promoter association of this complex. Consistently, the level of H3K4 methylation on PTEN promoter significantly increased upon AZD2281 treatment or PARP1 knockdown, and decreased upon doxorubicin treatment, further confirming that PAPR1 facilitates the binding of LSD1 at the PTEN promoter (**Figure 4A**). The ChIP samples were also analyzed by quantitative real-time PCR and similar results were obtained (**Figure 4B**). These results are not only supported by our earlier data showing that upon poly(ADP-ribosyl)ation of Snail, the Snail/LSD1 complex becomes stabilized (**Figure 3E**), but also in line with the notion that Snail

cooperates with LSD1 to downregulate PTEN in response to DNA damage, in such way that Snail fulfils its function as a survival factor.

PARP1 inhibitor AZD2281 enhances the killing effect of doxorubicin on cancer cells

Consistent with the results that doxorubicin enhanced the binding of the Snail-LSD1 complex at the PTEN promoter, we found that the protein level of PTEN was decreased in MDA-MB-157 and HCT116 cells upon doxorubicin treatment (lane 3, **Figure 5A**). Also as expected, the level of Akt phosphorylation was increased by doxorubicin. In contrast, AZD2281 treatment had the opposite effect on PTEN expression as well as Akt phosphorylation (lane 2, **Figure 5A**). Strikingly, when cells were treated with the two drugs simultaneously, the effect of doxorubicin on PTEN suppression as well as Akt activation (phosphorylation) was compromised by AZD2281 (lane 4, **Figure 5A**). To further test the idea that cancer cells apply a Snail complex-mediated defensive mechanism to evade DNA damage-induced apoptosis, we applied doxorubicin in combination with AZD2281 to cancer cells and examined their viability. As seen in Figure 5B, either doxorubicin or AZD2281 treatment can reduce proliferation of MDA-MB-157 and HCT116 cells; this suppressive effect was further decreased upon treatment of both drugs, indicating that the drug combination has enhanced the tumor suppressive effect. Taken together, our results suggest that blocking the activity of PARP1 can overcome the effect of doxorubicin on PTEN suppression and Akt activation, and sensitize cancer cells to the inhibitory/cytotoxic effect of doxorubicin.

DISCUSSION

In this study, we demonstrated that through interacting with and poly(ADP-ribosyl)ating Snail, PARP1 promotes the formation of Snail-LSD1 repressive complex and enhances the binding of the complex at the PTEN promoter for transcription suppression. Previously we have shown that Snail uses its highly conserved SNAG domain as a histone mimicking "hook" to recruit LSD1 to its target gene promoters[18]. The involvement of PARP1 in this regulation process particularly under the condition of DNA damage adds another layer to this delicate transcriptional machinery. As the founding member of the PARP superfamily, PARP1 is a multifunctional protein that not only plays a role in DNA repair, but also participates in gene transcription regulation. The effect of PARP1 could either be stimulatory or inhibitory, depending on the specific environmental context and cellular signals. In the very case discussed here, PAPR1 provides a stimulatory effect on the Snail-LSD1 complex under DNA damage. Upon activation by doxorubicin, PARP1 uses its pADPr for association with the pADPr-binding motif of Snail, and furthermore promotes the interaction of Snail with LSD1. Disruption of the pADPr-binding motif by point mutation not only resulted in loss of Snail-PARP1 association, but also strikingly compromised Snail-LSD1 complex formation. Consistently, blocking the degradation of pADPr by inhibiting PARG could enhance Snail-LSD1 interaction. In addition, we found that Snail could undergo poly(ADP-ribosyl)ation on DNA damage condition. Based on these results, together with previous finding that Snail interacts with LSD1 through its SNAG domain, we reasoned that binding and modification of Snail by PARP1 could change the conformation of Snail and potentially expose its LSD1-binding motif on the SNAG domain to facilitate Snail-LSD1 interaction. Therefore, LSD1 can be recruited by Snail to the target gene (PTEN in this case) promoter, where it demethylates histone H3 lysine 4 in favor of transcription repression. The model provided here illustrated a tantalizing mechanism of how cancer cells defense themselves against DNA damage and try to evade PTEN-mediated apoptosis (**Figure 5C**). A detailed computer-based structure analysis would hopefully further illustrate this dynamic regulatory process and will be done in the near future. We also tried to explore our findings by specifying the residues on Snail protein that are subject to poly(ADP-ribosyl)ation. Mutation of the lysine residue on the pADPr-binding motif of Snail did not significantly compromise the level of poly(ADP-ribosyl)ation, neither did mutations on Lys9, Asp12 or Lys16 of SNAG domain (data not shown), indicating that Snail can undergo poly(ADP-ribosyl)ation on multiple residues, which remain to be defined in the future. Together, our study illustrated the cooperation of Snail, LSD1 and PARP1 in PTEN transcription suppression under DNA damage condition. Previous to our study, there have been reports demonstrating the dynamic roles of PARP1 in gene transcription regulation. For example, upon binding to nucleosomes, PARP1 may regulate the compaction/decompaction of chromatin[35, 36]; PARP1 may also exclude histone H1 from its target gene promoters in favor of

transcription activation[37]. In addition, PARP1 has been shown to interact noncovalently with DNA methyltransferase 1 (Dnmt1) and inhibit its enzymatic activity, in such way that it regulates genomic methylation patterns[38]. As another typical example, PARP1 can dissociate Smad complexes from DNA through poly(ADP-ribosyl)ating Smad3 and Smad4, therefore attenuating Smad-mediated transcription and inhibiting TGF-β-induced epithelial-mesenchymal transition (EMT)[39]. Since we have demonstrated before the function of Snail-LSD1 on E-cadherin suppression and EMT induction, there is also a possibility that PARP1 participates in this regulation process. In contrast to the Smad-PARP1 model, however, poly(ADP-ribosyl)ation of Snail by PARP1 would rather enhance the inhibitory effect of Snail-LSD1 on E-cadherin and promote EMT. We reason that in different biological settings, cells may rely on different transcriptional machineries to turn on or off the expression of specific genes in response to various signals, or for the adaptation of different extracellular and intracellular stresses. Our hypothesis is further supported by recent finding that PARP1 can poly(ADP-ribosyl)ate and stabilize Snail and promote Snail-mediated EMT[40]. We speculate that PARP1 regulates Snail-mediated EMT using a more complicated and delicate mechanism, probably through promoting the recruitment or release of other histone-modifying enzymes and cofactors (LSD1 for example) to further modify chromatin. Since both E-cadherin and PTEN loss can promote the generation of cancer stem cells, another intriguing question is to what extent the Snail-LSD1-PARP1 complex contributes to cell stemness. While more work is still needed for clarification of the whole mechanism, our current study provides important information for the completion of the model of Snail-mediated transcription regulation.

The second insight provided by our study lies in the finding that PARP inhibitors in conjunction with DNA-damaging reagents may represent an effective treatment strategy against a much wider range of cancers. While the conventional chemotherapeutic drugs such as doxorubicin function by targeting DNA synthesis and cell division, unfortunately they are not smart in pinpointing cancer cells; rather they also do harm to normal cells with rapid dividing property. Even worse, many solid tumors continually undergoing chemotherapy will ultimately acquire drug resistance. Many cancer cells have defective DNA repair pathways. For example, BRCA1/2 mutations are found in breast and ovarian cancers[41, 42], and mutations of ataxia telangiectasia mutated (ATM) gene are identified in lymphoid malignancies[43]. In this regard, targeting DNA repair machineries is a promising strategy for achieving synthetic lethality on cancer treatments. Indeed, PARP inhibitors are prevailingly considered as treatment options against BRCA1/2-deficient tumors due to the synthetic lethality effect[30-32]. Under the same rationale, PARP1 inhibitors are also used in the treatment of tumors deficient in PTEN, which plays a critical role in the expression of the repair protein RAD51[44]. Currently, different PARP inhibitors combined with DNA-damaging reagents are under investigation in several clinical trials[45]. We have shown the enhanced suppressive effect of

doxorubicin-AZD2281 combination on BRCA1/2 and PTEN wild-type MDA-MB-157 and HCT116 cells. Based on our results, we argue that in addition to the induction of DNA damage, doxorubicin treatment also enhances Snail-LSD1 mediated PTEN suppression in a PARP1-dependent manner, which results in phosphorylation and activation of pro-survival Akt. Inhibition of PARP1 can compromise this undesirable effect while synergizing the DNA-damaging effect of doxorubicin to efficiently suppress tumor cells. While *in vivo* experiments are required to consolidate our results as well as to evaluate the long-term effect of PARP1 inhibition, our data expands potential therapeutic benefits of PARP1 inhibitors, especially on tumors with high levels of Snail and LSD1 expression. Furthermore, it is inviting to see if PARP1 inhibitors can synergize with LSD1 inhibitors and novel SNAG domain-mimicking compounds that block Snail-LSD1 interaction to treat these kinds of cancers[46].

In summary, we demonstrated that PARP1-mediated poly(ADP-ribosyl)ation of Snail is critical for Snail-LSD1 complex formation and the downstream PTEN suppression. Our study not only provides a new insight into the working mechanism of the Snail transcriptional machinery, but also explores the potential application of PARP inhibitors in conjunction with DNA damage-inducing reagents in targeting cancer cells. As PARP inhibitors are thrust into the limelight by the encouraging results of early clinical trials, our study would provide extra impetus for future drug development and help to diversify cancer treatment strategies.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

Human cDNA for PARP1 and LSD1 were amplified from HeLa cDNA and respectively cloned into pCMV-Tag2B with an N-terminal Flag tag. Snail mutant was generated using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) as described (Wu et al, 2009a). All sequences were verified by DNA sequencing. Antibodies against Snail, LSD1, PARP1, PTEN, Akt and Akt-P were purchased from Cell Signaling Technology (Danvers, MA); anti-H3K4me2 from Millipore (Bedford, MA); anti-Flag from Sigma-Aldrich (St Louis, MO); anti-HA from Roche Molecular Biochemicals (Indianapolis, IN); anti-pADPr from Trevigen (Gaithersburg, MD). Smartpool siRNA against human PARP1 was from Dharmacon. Doxorubicin was purchased from Sigma-Aldrich, Gallotannin from Santa Cruz, and AZD2281 from Selleck Chemicals.
Cell cultures and transfections

The human embryonic kidney HEK293, breast cancer MDA-MB157 and colon cancer HCT116 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's/F12 medium plus 10% fetal bovine serum as described previously (Wu et al, 2009a). Plasmids were transiently transfected into cells using FuGENE 6 (Roche Molecular Biochemicals).

Western blot analysis and immunoprecipitation

For protein extraction, 5×10^5 cells per well were plated onto six-well plates, before protein extraction and western blot analysis. For immunoprecipitation, cells were lysed in buffer (50 mmol/l Tris (pH 7.5), 150 mmol/l NaCl, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 1% NP-40, 1 mmol/l EDTA and 0.25% deoxycholate). Total cell lysates (1000 μg) were incubated overnight with desired antibodies conjugated to agarose beads (Roche Molecular Biochemicals) at 4°C. The beads were then washed with PBS, and the immunoprecipitated protein complexes were resolved by 10% SDS–PAGE.

Chromatin immunoprecipitation

ChIP assays were performed according to the protocol described by Nowak et al (2005) with some modifications. The cells were crosslinked with disuccimidyl glutarate (Pierce, Rockford, IL) and formaldehyde at room temperature. Cells were subjected to lysis with L1 buffer (50 mM Tris, 2 mM EDTA, 0.1% IGEPAL, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (pH 8.0)) on ice. After centrifugation, the nuclear pellet was resuspended in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris and protease inhibitor mixture (pH 8.0)). Cell lysates were subjected to sonication and then incubated with 4 μg of Snail1 (Abcam), LSD1 (Sigma-Aldrich) or H3K4me2 antibody overnight, followed by incubation with a 50% slurry of protein A–agarose/Salmon

sperm DNA (Upstate Biotechnology, Lake placid, NY) for 3 h at 4°C. Bound DNA–protein complexes were eluted and crosslinks were reversed after a series of washes. Purified DNA was resuspended in TE buffer (10 mM Tris–HCl and 1 mM EDTA (pH 8.0)) for PCR. The primers for the PTEN promoter were 5′-CCGTGCATTTCCCTCTACAC-3′ and 5′-GAGGCGAGGATAACGAGCTA-3′.

Real-time quantitative PCR

Quantitative real-time PCR experiments were performed using SYBR Green Power Master Mix following the manufacturer's protocol (Applied Biosystems).

MTT assay

MTT assays were performed using standard protocol. Cell count and incubation time were optimized. Assays were repeated three times.

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FIGURE LEGENDS

Figure 1. **Doxorubicin enhances PAPR1-Snail interaction**. (**A**) The Snail complex was isolated from the stable HEK293 cells expressing Snail. The complex were separated on SDS–PAGE and visualized by silver staining. LSD1 and PARP1 were identified by mass spectrometry. (**B**) Flag-tagged PARP1 and HA-tagged Snail were co-expressed in HEK293 cells. Cells were treated with 1 μM of Doxorubicin (DOX) for 6 hours before harvesting. After immunoprecipitation of PARP1, bound Snail was examined by western blotting. (**C**) MDA-MB157 and HCT116 cells were treated with Doxorubicin as described above. After immunoprecipitation of PARP1 endogenous PARP1, bound endogenous Snail was examined by Western blotting.

Figure 2. **PAPR1 positively regulates Snail-LSD1 interaction**. (**A**) Flag-tagged LSD1 and HA-tagged Snail were co-expressed in HEK293 cells. After immunoprecipitation of LSD1, bound Snail was examined by Western blotting. For comparison, cells were either co-expressed with Flag-tagged PAPR1 or treated with 1 μM of doxorubicin 6 hours before harvesting cells. (**B**) Endogenous LSD1 was immunoprecipitated from MDA-MB157 and HCT116 cells and bound endogenous Snail was examined by Western blotting. For comparison, cells were treated with doxorubicin (1 μM), AZD2281 (2μM), or transfected with PARP1 siRNA.

Figure 3. **Snail contains a potential pADPr-binding motif and is subject to poly(ADP-ribosyl)ation**. (**A**) Sequence alignment of Snail with previously established pADPr-binding motif. The conserved residues were highlighted with red color. (**B**) Flag-tagged PARP1 was co-expressed with HA-tagged wild-type (WT) or mutant (R151A/K152A) Snail in HEK293 cells. Cell were treated with or without doxorubicin. After immunoprecipitation of PARP1, the bound Snail was examined. (**C**) Flag-tagged LSD1 was co-expressed with HA-tagged WT or mutant Snail. After immunoprecipitation of LSD1, the bound Snail was examined. (**D**) Flag-tagged LSD1 was co-expressed with HA-tagged Snail. Cell were treated with 10 μM of gallotannin (GN) for 6 hours. After immunoprecipitation of PARP1, bound Snail was examined. (**E**) WT or mutant Snail was expressed in HEK293 cells and treated with 10 mg/ml of cycloheximide (CHX) for different time intervals. The level of Snail was analyzed by Western blotting. Densitometry results were statistically analyzed and plotted (bottom panel, mean ± SD from 3 separate experiments). A representative blot is shown in the top panel. (**F**) HKE293 cells stably expressing Snail-HA were treated with doxorubicin and AZD2281. After Snail was immunoprecipitated, poly(ADP-ribosyl)ation of Snail was analyzed by Western blotting using antibody against pADPr.

Figure 4. **The enzymatic activity of PARP1 is required for Snail-LSD1 binding to PTEN promoter**. (**A**) MDA-MB157 and HCT116 cells were treated with doxorubicin or AZD2281, or transfected with PARP1 siRNA. The association of endogenous Snail and LSD1 with the PTEN promoter was analyzed by ChIP assay. Methylation of H3K4 on the PTEN promoter was also analyzed by ChIP assay using antibody against H3K4me2. (**B**) The ChIP samples were analyzed by quantitative real-time PCR (mean ± SD from three separate experiments).

Figure 5. **AZD2281 enhances the killing effect of doxorubicin on cancer cells**. (**A**) MDA-MB157 and HCT116 cells were treated with AZD2281, doxorubicin, expression of PTEN, Akt and phosphorylated Akt (Akt-P) was examined by Western blotting. (**B**) The proliferation of cells in (A) were analyzed by MTT assays (mean ± SD from 3 separate experiments). **(C)** A proposed model illustrating that Snail recruits LSD1 to PTEN promoter in a PARP1-dependent manner. Under DNA damage condition, Snail becomes tightly associated with PARP1 and is subject to PARP1-mediated poly(ADP-ribosyl)ation, which promotes the interaction of Snail with LSD1, resulting the recruitment of LSD1 at the PTEN promoter for transcription repression. Inhibition of PARP1 facilitates the growth suppressive effect of doxorubicin by restoring PTEN expression.

Figure 1

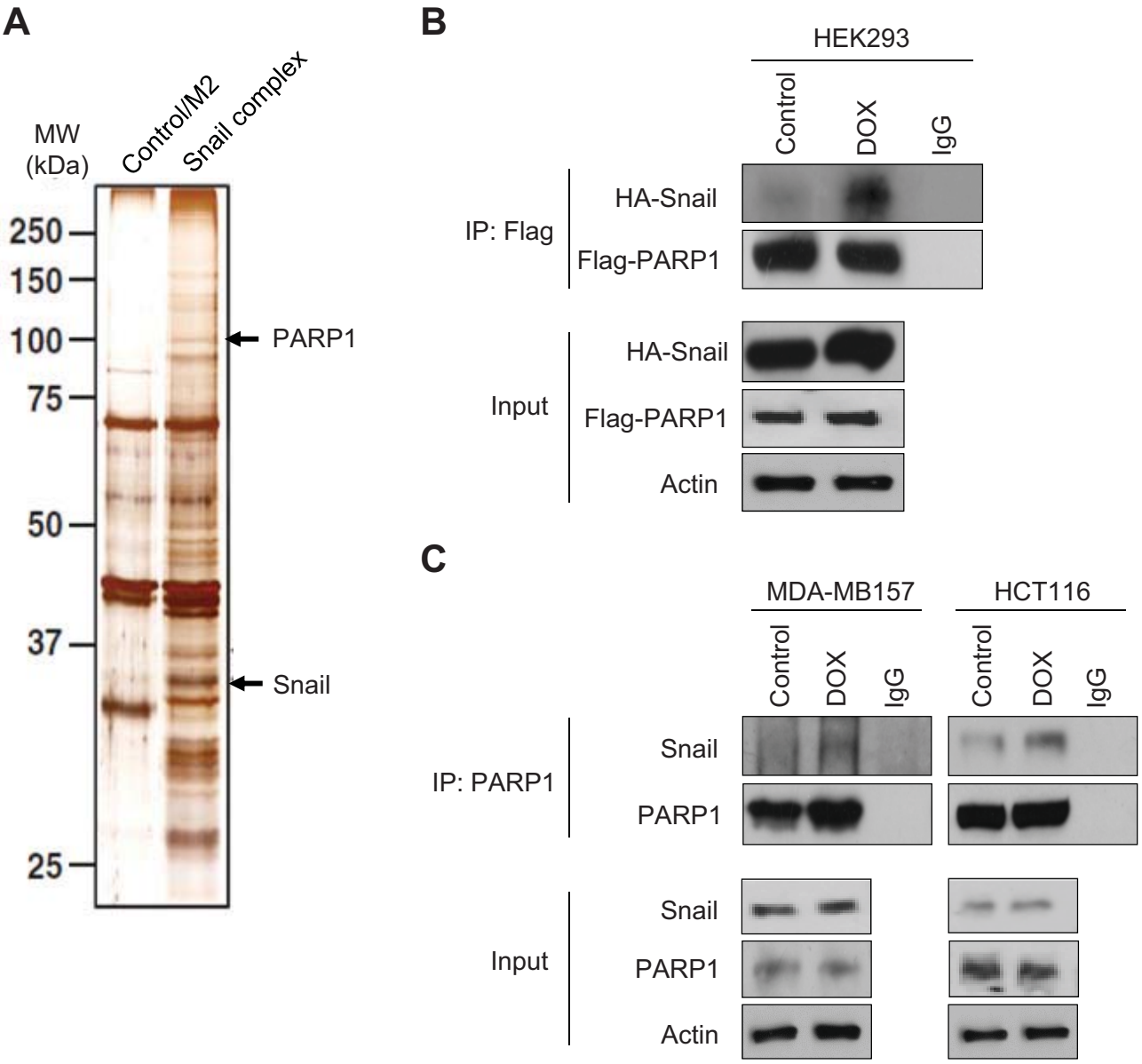
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Figure 2

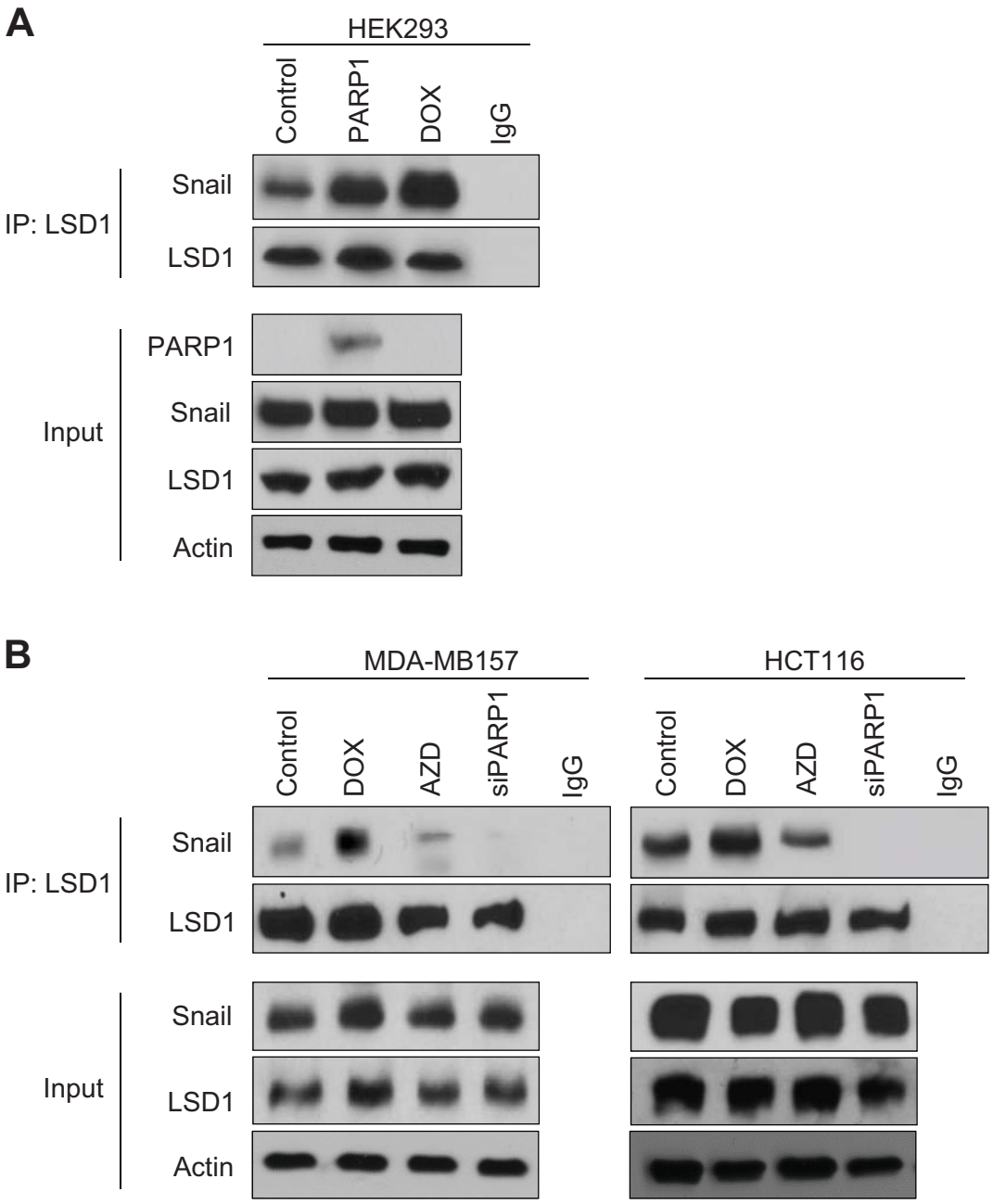
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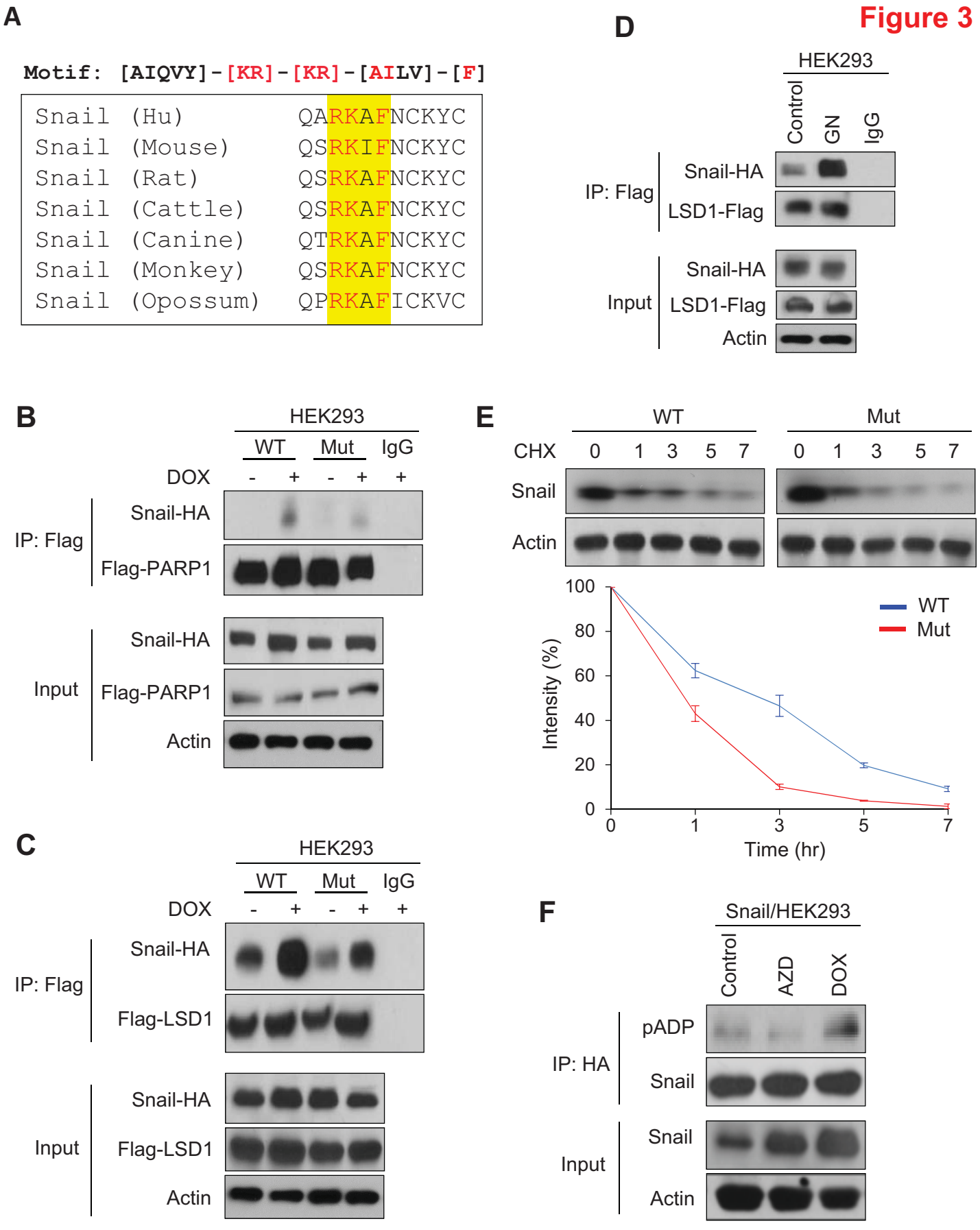
Figure 3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Snail (Mouse) QSRKIFNCKYC
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Snail (Cattle) QSRKAFNCKYC
Snail (Canine) QTRKAFNCKYC
Snail (Monkey) QSRKAFNCKYC
Snail (Opossum) QPRKAFICKVC
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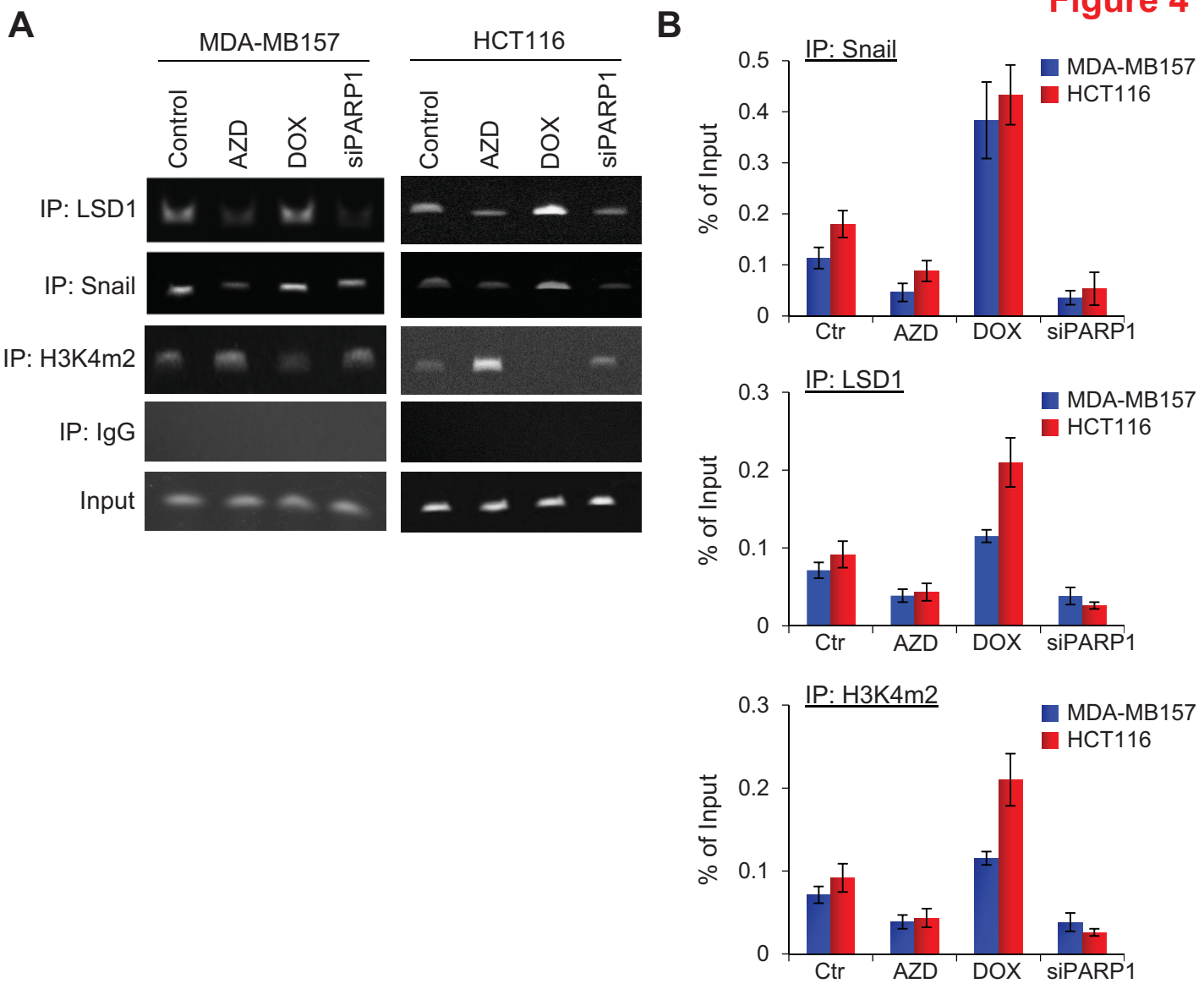
Figure 4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IP: LSD1
IP: H3K4m2
MDA-MB157
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% of Input
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Ctr
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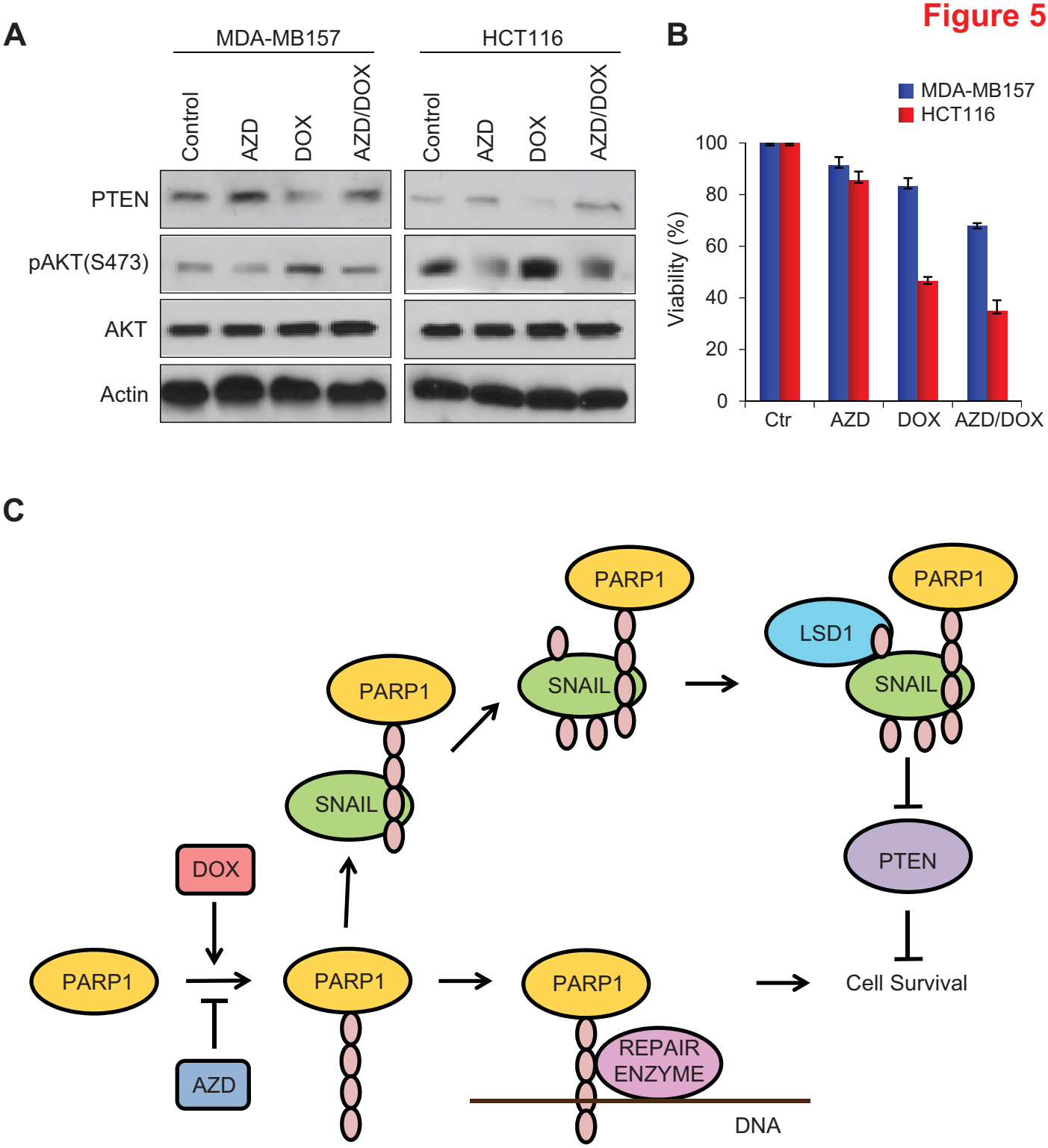
Figure 5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Viability (%)
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80
60
40
20
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Ctr
AZD
DOX
AZD/DOX
C
PARP1
SNAIL
LSD1
PTEN
DOX
AZD
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Epigenetic Regulation of EMT: The Snail Story

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Abstract: While the epithelial-mesenchymal transition (EMT) plays a fundamental role during development, its deregulation can adversely promote tumor metastasis. The phenotypic and cellular plasticity of EMT indicates that it is subject to epigenetic regulation. A hallmark of EMT is E-cadherin suppression. In this review, we try to embrace recent findings on the transcription factor Snail-mediated epigenetic silencing of E-cadherin. Our studies as well as those of others independently demonstrated that Snail can recruit various epigenetic machineries to the E-cadherin promoter. Based on these results, we propose a model of epigenetic regulation of EMT governed by Snail. Briefly, recruitment of the LSD1/HDAC complex by Snail facilitates histone H3K4 demethylation and H3/H4 deacetylation. Histone deacetylation may promote subsequent recruitment of PRC2 to methylate H3K27, while H3K4 demethylation favors the association of H3K9 methyltransferases G9a and Suv39H1. Finally, DNA methyltransferases (DNMTs) can be recruited to the promoter area in a G9a/Suv39H1-dependent manner. Together, these chromatin-modifying enzymes function in a Snail-mediated, highly orchestrated fashion to suppress E-cadherin. Disruption of the connection between Snail and these epigenetic machineries may represent an efficient strategy for the treatment of EMT-related diseases, including tumor metastasis.

Keywords: EMT, epigenetic regulation, Snail.

EMT IS SUBJECT TO DYNAMIC EPIGENETIC REGULATION

As a commanding cellular event during embryogenesis and tumor metastasis, epithelial-mesenchymal transition (EMT) has been a long-time research interest in the field of molecular biology. Phenotypically, upon receipt of microenvironmental stimuli such as tumor growth factor β (TGF-β), Wnt and tumor necrosis factor-α (TNF-α) [1-5], epithelial cells get ripped off their ability to adhere to each other, and lose their apical-basal polarity; in the meanwhile, they gain mesenchymal cell characteristics such as increased motility, invasiveness and resistance to apoptosis [6-8]. While EMT was originally recognized as a feature of mesoderm formation in both *Drosophila* flies and mammals [9, 10], it was later revealed to play crucial roles in tumor metastasis. Indeed, there has been strong evidence suggesting that EMT is an early event during invasion and metastasis of many carcinomas [11, 12]. Of note, EMT confers tumor cells with stem cell-like properties, as evidenced by the expression of stem cell markers, and acquisition of mammosphere-forming, self-renewal and differentiation capabilities, all of which account for immunosuppression and tumor recurrence [13-16]. Remarkably, EMT is a reversible process, with its counterpart mesenchymal-epithelial transition (MET) functioning to restore tumor cells with epithelial characteristics following their dissemination and distant colonization, again under the guidance of extrinsic signals. The phenotypic and cellular plasticity of EMT is reminiscent of involvement of an epigenetic regulation program [17].

The epigenetic code, which was introduced to denote the determinants of gene features other than DNA sequence [18-20], is maintained/regulated through a dedicated system highlighted by dynamic histone modifications and DNA methylation [21-23]. While histone modifications generate reversible local chromatin structures, DNA methylation provides reinforcement as well as establishment of gene silencing by keeping chromatin in a relatively stable long-term repression state [22]. Among the diversified histone modifications, acetylation and methylation, as well as their counterparts, i.e. deacetylation and demethylation, play essential roles in conferring genes with specific transcriptional potential [21]. Histone acetylation and deacetylation are mediated by histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. While acetylation introduces an "open chromatin" state and increases the accessibility of transcription complexes, deacetylation does exactly the opposite to impede gene transcription [24]. In terms of histone methylation/demethylation, things become more complicated. For example, methylations on histone H3 lysine 4, 36 and 79 are generally considered as transcription activation marks, whereas methylations on histone H3 lysine 9 and 27 are linked to transcription repression [25]. Histone modifications are dynamically regulated by specific chromatin enzymes and their cofactors, which have been critically reviewed by others [26-30]. DNA methylation, on the other hand, is executed by a family of highly related DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b), which function by transferring a methyl group to the cytosine in a CpG dinucleotide. Typically, the maintenance of DNA methylation in somatic cells is attributed to DNMT1, whereas *de novo* DNA methylation during embryonic development is credited to DNMT3a and DNMT3b [31, 32]. While DNA methylation is executed by DNMTs, DNA demethylation was initially considered as a passive process, i.e., through DNMT suppression [32]. Not until recently has an active DNA demethylation mechanism been discovered, with the identification of ten-eleven translocation 1 (TET1) to be responsible for removing the 5'-methylcytosine (5 mC) during the DNA mismatch repair process [33-35].

It is established that DNA methylation and diverse histone modifications are interdependent events with specific hierarchies and/or feedback loops [22, 36-38]. On one hand, a specific histone modification pattern would predispose DNA methylation. Basically, as an initial step in gene repression, removal of methylation on histone H3 lysine 4 (H3K4) releases the inhibitory effect on the approaching of DNMT3a and DNMT3b to the nucleosome, therefore presetting a welcome environment for *de novo* DNA methylation to take place [39]. Subsequently, deacetylation of histone H3/H4 opens the door for the landing of histone methyltransferases such as G9a and SUV39H1, which use different mechanisms to further promote the recruitment of *de novo* DNA methyltransferases

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[22]. On the other hand, once the pattern of DNA methylation has been set, it serves as a template for the reconstruction of epigenetic state during the cell differentiation process. Indeed, there are previous studies demonstrating that methyl-CpG-binding domain proteins (MBDs) residing in the chromatin region with DNA methylation mediate the recruitment of HDACs; in addition, DNA methylation predisposes H3K9 dimethylation (H3K9me2) while excluding methylation on H3K4 [40, 41]. Overall, the epigenetic landmark is maintained in such a sophisticated fashion that disruption of the balance of epigenetic stage, either at the genome scale or even restricted to a certain gene, is usually the cause of many diseases, including cancer [42, 43]. Currently, many mechanistic details of the crosstalk between histone modifications and DNA methylation remain to be fully uncovered. An intriguing fact, for example, lies in that tumor cells harbor genome-wide DNA hypomethylation, which creates genomic instability due to the loss of transcription silencing of many transposons [32], whereas they usually have the tumor suppressor gene promoters hypermethylated compared to their normal siblings. It seems reasonable to argue that the DNA methylation machineries selectively target the promoters of tumor suppressor genes to silence their expression, therefore providing tumor cells with a growth advantage [22, 44]. Identification of the key molecules that mediate this selection process will undoubtedly help us better appreciate the epigenetic regulation program in cancer.

As mentioned, EMT, with its hallmark being loss expression of E-cadherin [7, 45], is intimately associated with an epigenetic regulation program. Indeed, in basal-like breast cancer (generally referred to as triple-negative breast cancer) that has typical EMT characteristics and high metastatic potential [46-50], DNA methylation at the E-cadherin promoter is not uncommon. What is the driving force for DNA methylation to take place at the E-cadherin promoter? Are there additional epigenetic events that happen before the long-term repression by DNA methylation takes effect? How can these events get organized into a hierarchic fashion to eventually boost promoter DNA methylation in specific biological context of EMT? To answer these questions, efforts should be made to decipher the potential built-in connection between histone modifications and DNA methylation during this dynamic cellular event.

EARLIER STUDIES INDICATE THAT SNAIL PLAYS CRUCIAL ROLES IN ASSEMBLING REPRESSOR COMPLEXES ON E-CADHERIN PROMOTER

Several transcription factors, such as Snail, Twist and ZEB1, have been recognized as E-cadherin suppressors and EMT inducers [6, 7]. Not surprisingly, these transcription factors are well known for their roles in early embryogenesis as well as tumor metastasis. Typically, the expression of the transcription factor Snail, which was originally identified in *Drosophila* as a suppressor of *shotgun* (an E-cadherin homologue), is essential for the formation of the mesoderm and neural crest, indicating its fundamental role in morphogenesis [51-53]. Furthermore, Snail expression correlates with tumor grade and nodal metastasis in invasive breast ductal carcinoma; and overexpression of Snail is indicative of poor clinical outcome in patients with breast cancer [11, 54-56]. As an ideal target for the study of EMT, Snail has been under extensive investigation for decades. However, not until recently has the linkage between Snail and epigenetic regulation of EMT been discovered.

According to the chromatin "reader and writer" idea, as a transcription factor, Snail is able to "read" its target gene (E-cadherin in this case) promoter using its DNA-binding zinc fingers; however, Snail does not have chromatin modulation activity and falls short of "writing" either DNA methylation or histone modifications by itself. It is hypothesized that transcription factors (as chromatin readers) cooperate with chromatin enzymes and cofactors (as chromatin writers) to modulate epigenetic information in favor of gene transcription activation or repression. Indeed this reader-writer model ideally fits in the case of Snail. Rapidly emerging were study results convincingly demonstrating that Snail forms complexes with and then recruits different chromatin modifying enzymes and cofactors to E-cadherin promoter for transcription repression.

Snail Associates with the SIN3 Repressor Complex Containing Histone Deacetylase (HDAC) Activity

All Snail family members share a highly conserved zinc finger domain at the C-terminal, which functions to bind DNA on a sequence-specific basis. Typically, the domain is comprised of four to six C_2H_2 type zinc fingers and recognizes the E-box 5'-CACCTG-3' present in the promoter region of E-cadherin, among others [52]. *Drosophila* Snail also contains an N-terminal binding domain for the corepressor CtBP (C-terminal binding protein); upon binding, Snail can recruit CtBP to its target gene promoters for transcription repression [52, 57]. In the case of Vertebrate Snail proteins, namely Snail, Slug and Smuc, they share a conserved SNAG (Snail/Gfi) domain at the N-terminal, which contributes to their repressive function [52].

The first evidence for the interaction of the SNAG domain with repressors came from studies of Peinado and colleagues. They demonstrated that mouse Snail uses its SNAG domain to associate with HDAC1/2 and corepressor mSin3A, and recruits the repressor complex to E-cadherin promoter, where HDAC1/2 deacetylate histone H3 and H4 to create the repressive chromatin environment [58]. Treatment of cells with histone deacetylase inhibitor Trichostatin A (TSA) efficiently abolished the repressive effect of Snail [58]. Based on previously studies demonstrating the connection and mutual communication between histone deacetylation and DNA methylation [59-62], they hypothesized that Snail recruits HDACs to E-cadherin promoter for histone deacetylation, which functions to maintain DNA methylation and gene silencing. Furthermore, Peinado and colleagues found that the level of histone H3 lysine 9 dimethylation (H3K9me2), which is a transcription repression mark, was increased at the E-cadherin promoter region upon overexpression of Snail in epithelial cells, indicating the involvement of other repressor complexes containing histone methyltransferase activities during the regulation process [58].

Snail Cooperates with Polycomb Repressive Complex 2 (PRC2) for Histone H3 Lysine 27 Trimethylation (H3K27me3)

Herranz and colleagues have recently demonstrated the recruitment of PRC2 by Snail to E-cadherin promoter, providing a second example for Snail-mediated epigenetic regulation machinery [63]. Polycomb proteins (PcG) play fundamental roles on chromatin remodeling and gene silencing during embryonic development as well as stem cell differentiation [64-66]. As one of the PcG complexes, PRC2 contains methyltransferase activity on histone H3 lysine 27, and functions to initiate the transcription silencing process [67]. Herranz and colleagues found that PRC2 was required for Snail-mediated E-cadherin suppression, as knockdown of essential PRC2 components Suz12 or Ezh2 abolished the suppressive effect of Snail in pancreatic cancer cell line RWP-1 as well as colon adenocarcinoma cell line SW-620 [63]. While the interaction between Snail and PRC2 could be direct or indirect, they demonstrated the necessity of the SNAG domain of Snail for recruiting PRC2 [63]. Other than their studies, researches from two independent groups showed that the SNAG domain of Snail could interact with both the Ajuba LIM and PRMT5 proteins, the latter of which then associated with Suz12 through a mediator MEP50 [68, 69]. Based on these results, it seems more than likely that the SNAG domain of Snail recruits PRC2 in an Ajuba/PRMT5-dependent fashion. Although not supported by comprehensive data, Herranz and colleagues suggested another possibility that the interaction of Snail with PRC2 is mediated by HDACs, according to the facts that HDACs can associate with both Snail and PRC2, and that histone deacetylation supports PcG-mediated transcription suppression [70, 71].

Together, earlier studies have strongly indicated the crucial role of Snail on epigenetic silencing of E-cadherin, typically through recruiting multiple repressor complexes to the E-cadherin promoter region. How does Snail efficiently organize these epigenetic machineries, allowing the regulation events to continue smoothly to the final step of inhibition, i.e. promoter DNA methylation? Is there any correlation between the expression of Snail and those of chromatin enzymes in tumor cells that are undergoing EMT? And more importantly, how can the Snail story help us rationalize epigenetic drug design to efficiently rectify EMT deregulation? Based on extensive experiments as delineated in the following part, we are trying to draw a conclusive remark on the Snail-mediated epigenetic network.

SNAIL LINKS TRANSCRIPTIONAL CONTROL OF E-CADHERIN WITH EPIGENETIC REGULATION DURING EMT

Snail Uses its SNAG Domain as a Molecular Hook for Recruiting Histone Lysine-specific Demethylase 1 (LSD1) Complex

We recently applied an unbiased protein affinity purification-mass spectrometry coupled technology to identify Snail-interacting proteins using HEK293 cells stably expressing dual-tagged human Snail [72]. Among the Snail partners, we found several interesting candidates, including LSD1, G9a, and Suv39H1. As the first identified histone demethylase, LSD1 forms a repressor complex together with HDAC1/2, CoREST and BHC80 [73], and functions by specifically removing methylation marks on H3K4 and initiating the transcription repression process [74]. Previous studies have established that LSD1 can cooperate with different transcription factors to selectively repress gene transcription [75-78]. Most importantly, LSD1 plays an essential role during development, and overexpression of LSD1 has been correlated with malignant progression of multiple cancers, including primary neuroblastic tumors, prostate cancer, and ER-negative breast cancer [79-81].

According to our preliminary results, we speculated that Snail and LSD1 form a repressor complex to downregulate E-cadherin and induce EMT. Indeed, we found that the SNAG domain of Snail and the amine oxidase (AO) domain of LSD1 are responsible for protein interaction [72]. Strikingly, we noticed that the sequence of the SNAG domain highly resembles that of histone H3 tail, and both of them are rich in positively charged lysine and arginine residues. Computer modeling analysis further revealed that the SNAG domain can adopt a conformation that is superimposed by the histone tail and bind the enzymatic cleft of LSD1 with high affinity [72]. We dug further to find that Arg3, Arg8 and Lys9 of the SNAG domain participate in critical contacts with LSD1, similar to the case of their counterparts Arg2, Arg8 and Lys9 of histone H3. Based on our studies, we proposed a model in which Snail uses its histone H3-mimicking SNAG domain as a molecular "hook" (or pseudo-substrate) to recruit LSD1 and potentially other molecules such as HDAC1/2 and the corepressor CoREST to the E-cadherin promoter, where LSD1 demethylates histone H3K4 and HDACs deacetylate both histone H3 and H4 to initiate the transcription repression process [72]. Consistently, Baron and colleagues performed a more detailed structural analysis on Snail-LSD1 binding and found that the SNAG domain and histone H3 tail both harbor positively charged groups and hydroxyl side chains, which enable them to fit into the catalytic cavity of LSD1 in a similar conformation [82]. Furthermore, we performed functional studies to determine the role of Snail and LSD1 during EMT. Knockdown of Snail and LSD1 expression can significantly suppress the migration and invasion activities of colon cancer cell line HCT116, as well as breast cancer cell lines PC3 and MDA-MB231, which further consolidate our findings of the role of the Snail-LSD1 duo in EMT induction [72].

Snail Recruits Histone H3K9 Methyltransferase G9a and Suv39H1 to Further Promote DNA Methylation

Methylation on H3K9 is a well-conserved epigenetic mark for heterochromatin formation and transcriptional silencing [83, 84]. Among various histone methyltransferases, G9a is responsible for H3K9 mono- and dimethylation (H3K9me1 and H3K9me2) at euchromatin and facultative heterochromatin [85, 86]. Our recent study demonstrated that Snail interacted with G9a both *in vitro* and *in vivo* and was required for the enrichment of G9a and corresponding H3K9me2 at the E-cadherin promoter. The domains responsible for their interaction were mapped to the ankyrin-repeat and SET domains of G9a and C-terminal zinc-finger region of Snail, respectively [87]. As mentioned, DNA methylation is executed by DNMTs and commonly occurs in the promoter region of genes [31, 32]. According to our study, Snail can also interact with DNMT1, DNMT3a and DNMT3b, and these interactions are likely to be indirect, as knockdown of G9a expression disrupted the interaction of Snail with DNMTs. Interestingly, H3K9me2 coincided with DNA methylation at the E-cadherin promoter, and knockdown of G9a expression significantly inhibited DNA methylation at the promoter region and reactivated E-cadherin expression in MDA-MB-231 breast cancer cells. This is consistent with previous studies demonstrating that DNA methylation can be remarkably affected in G9a knockout embryonic cells [88]. Also in line with our findings, G9a is enriched at the promoters of aberrantly methylated genes in multiple cancer cells, and co-recruitment of G9a, DNMT1, and HP1 to the promoter of the survivin gene stimulates H3K9me2 and DNA hypermethylation [89]. Together, our study as well as those of others suggests that G9a-mediated H3K9me2 is one of the key events in the maintenance of silencing gene promoters in cancer.

Another Snail-interacting protein, Suv39H1, is a histone methyltransferase responsible for the trimethylation of H3K9, which is typically associated with constitutive heterochromatin. Our results indicated that the SNAG domain is required for the association of Snail with Suv39H1, with amino acid residues Phe5, Lys9, and Ser11 within the domain playing key roles in the protein interaction. Furthermore, we found that the interaction of Suv39H1 with Snail is critical for H3K9 trimethylation (H3K9me3) on the E-cadherin promoter in breast cancer cells. According to previous studies, H3K9me3 catalyzed by Suv39H1 commonly links to *de novo* gene silencing by promoting promoter DNA methylation. Indeed, DNA methylation at major satellite repeats is apparently decreased in Suv39H1/Suv39H2 double-knockout embryonic stem cells [90]. In addition, it has been demonstrated that Suv39H1 creates an H3K9me3 binding and docking site for the adaptor molecule HP1, which in turn recruits DNMT3b and HDAC to catalyze DNA methylation and histone de-acetylation, respectively [37]. Consistent with these findings, we showed that increased H3K9me3 is accompanied by decreased acetylation of H3K9 and increased DNA methylation on the E-cadherin promoter, and knockdown of Suv39H1 can reverses these events and re-activate E-cadherin expression, indicating the critical role of Suv39H1 during Snail-mediated E-cadherin suppression.

The interaction of Snail with G9a and Suv39H1 gave us a couple of implications. Methylation on H3K9 is a sequential process, with the lysine residue subject to modifications of up to three methyl groups. While G9a catalyzes the mono- and dimethylation processes, Suv39H1 uses the product of G9a, i.e. H3K9me, as a substrate, and further converts it into H3K9me3 [91, 92]. We reasoned that G9a and Suv39H1 cooperate to establish the silencing heterochromatin at the E-cadherin promoter, typically with the help of Snail. Indeed, G9a, Suv39H1 and SETDB1, which is a H3K9 mono-methyltransferase, can assemble to form a giant complex in both euchromatin and heterochromatin areas, and the protein stability of one enzyme may affect the stability of the other two components [93]. Furthermore, there seems to be two distinct routes for H3K9 methyltransferases to promote DNA methylation

at the E-cadherin promoter. In the first route, G9a directly interacts with DNMTs and recruits them to the E-cadherin promoter through the association with Snail. In the second route, both G9a and Suv39H1 relies on their catalytic activities to respectively create H3K9me2 and H3K9me3, with both marks being able recruits HP1 and DNMTs to the promoter area [31]. This dual-mode of regulation may provide a double safety to guarantee the efficient methylation of E-cadherin promoter.

Snail Coordinates Histone Modifications and DNA Methylation: Conclusive Mark and Perspective

Based on our results as well as those of others, it becomes clear that Snail manipulates epigenetic outputs and masters EMT in a multi-step fashion. First, Snail uses its molecular hook, i.e. the SNAG domain, to associate with the LSD1/HDAC complex. Once recruited to the E-cadherin promoter region, the complex exerts its histone-modifying activities to create the initial repressive chromatin environment, i.e. H3K4 demethylation and H3/H4 deacetylation. Previous studies indicated that H3K9me2/3-enriched histones devoid of H3K4me2/3 and histones depleted of H3K4me2/3 have elevated H3K9me2/3, due to the fact that H3K9 methyltransferases including G9A and Suv39H1 fail to bind and methylate H3K4me2/3 substrates [94, 95]. We reasoned that demethylation of H3K4 by LSD1 provides a condition that favors Snail-mediated association of G9a and Suv39H1 on the E-cadherin promoter. In addition, HDACs may mediate the interaction of PRC2 with Snail, therefore promoting PRC2 to land in the promoter region. Second, G9a associates with the C-terminal region of Snail and is brought to the promoter region for H3K9me2, while Suv9H1 is drafted by the SNAG domain to land in the chromatin and uses H3K9me2 as substrate to generate H3K9me3. Since H3K9 methylation and acetylation are considered mutually exclusive, the level of acetylation would be further decreased. Third, the landing of G9a and Suv39H1 on chromatin serves as an intermediate step that bridges the initial H3K4 demethylation to the last modulation event, as it promotes the recruitment of DNMTs to work on DNA methylation, a final touch for gene silencing. Collectively, a picture appears in which Snail coordinates multiple epigenetic events to function in great synergy to suppress E-cadherin and induce EMT (Fig. **1**).

Attention should be paid to the fact that Snail is a highly unstable protein and subject to sophisticated regulation by microenvironmental stimuli. As a few typical examples, (1) the inflammatory cytokine TNFα can activate the NF-κB pathway to induce the expression of COP9 signalosome 2 (CSN2), which in turn blocks the ubiquitination and degradation of Snail [96]; (2) expression of stromal matrix metalloproteinase (MMP3) can increase cellular level of reactive oxygen species, resulting in Snail upregulation [97]; (3) adipose stromal cells surrounding tumors express aromatase as well as other estrogen metabolizing enzymes, therefore affecting the intratumoral estrogen levels and downstream signaling, including Snail upregulation and EMT induction [98]; and (4) platelet-derived TGF-β can induce SMAD and NF-κB signaling, both of which can lead to Snail expression [99]. Together, these studies suggest that Snail is deregulated under aberrant host microenvironment, which provides a suitable "soil" for EMT and metastasis to occur. While our study mainly focuses on the roles of Snail in human breast cancer metastasis, it should be noted that Snail protein is expressed in a variety of tissues including kidney, lung, placenta, heart, brain and liver. Previous studies from other groups indicated that Snail can induce EMT and promote metastasis of other human cancers including melanoma, pancreatic, colon, prostate and lung cancer [100-104]. Possibility exists that Snail cooperates with diverse epigenetic machineries during these processes. In the current review, we do not intend to provide a "one fit for all" model, as detailed mechanisms governing EMT may vary in specific tissues and cells. In addition to Snail, other transcription factors such as Slug, Twist and ZEB1/2 have been shown to recruit epigenetic machineries for E-cadherin suppression and EMT induction. For a comprehensive understanding of epigenetic regulation of EMT, further studies need to be done in the future. Collectively, we propose a model, in the typical example of Snail, that EMT is orchestrated by dynamic epigenetic events.

As our understanding on Snail-mediated EMT regulation continues to get improved, still many mechanistic details remain to be clarified. For example, after binding and bringing enzymes to chromatin, how will the SNAG domain get released to make room for histones? While we hypothesized that it is overabundant amount of histone proteins at the chromatin region that outcompetes the binding of SNAG, further experiments are required to address this issue. In addition, we are wondering whether the histone-mimicking SNAG domain can be modified during the recruitment process. Development of antibodies specifically recognizing SNAG domain with different modifications would definitely help to clarify this question. Once specific modifications on SNAG are confirmed, we can take one step further to find out how these modifications facilitate/obstruct the following epigenetic events during EMT. Chan and colleagues recently applied a pull-down approach in combination with LC-MC/MC analysis to successfully identify nuclear proteins that associate with histone H3 peptides harboring different lysine modifications [105]. Given the resemblance between the histone H3 tail and the SNAG domain, there seems to be a good chance to identify interacting proteins for diversely modified SNAG using a similar strategy. As a third enigma, while PRC2-mediated H3K27me3 is associated with gene repression, its enrichment is unexpectedly anti-correlated with DNA methylation [106, 107]. It is hypothesized that the expression of some developmentally important genes is governed by an "epigenetic switching" reprogramming event. Basically, these genes are repressed by PRC2 in normal cells, whereas they are subject to hypermethylation in cancer [108]. While the specific mechanism remains unclear, this hypothesis is compatible with our model that PRC2 recruitment precedes the approaching of DNMTs during cancerous EMT, and may partially explain the reduced epigenetic plasticity and permanent silencing of E-cadherin in multiple cancers. Future studies are required to clarify the role of PRC2 in this epigenetic modulation cascade.

Last but not least, the refreshing Snail story not only reminds us of the integrity and plasticity of epigenetic network, but also helps us for the development and optimization of epigenetic therapies targeting EMT-related diseases. As early as 2004, Azacitidine (trade name Vidaza), a potent inhibitor of DNA methyltransferases, got approval from FDA and became the first epigenetic drug on the market to treat bone-marrow cancer and blood cancer cancer [109]. Since then, epigenetic drugs emerge as novel treatment strategies. Currently, DNA methyltransferases and histone deacetylases remain the primary targets for epigenetic therapy. For instance, Vorinostat (formerly known as SAHA) is a histone deacetylase inhibitor used to treat the rare cancer cutaneous T cell lymphoma (CTCL) [110]. Interestingly, according to a recent study, application of a novel histone deacetylase inhibitor panobinostat (LBH589) can alter the expression of EMT markers in hepatocellular carcinoma models [111]; another study demonstrated the DNA methylation inhibitor Decitabine (trade name Dacogen) as well as HDAC inhibitors Sodium 4-phenyl butyrate and Trichostatin A can change the morphology and the expression of differentiation markers in acute myelogenous leukemia (AML) cells [112]. Beyond targeting DNA methyltransferases and histone deacetylases, there are plenty of preclinical studies underway for evaluation of LSD1 inhibitors on multiple types of cancers [81, 113, 114]. Most recently, Schenk and colleagues applied LSD1 inhibitor tranylcypromine (TCP) in combination with all-trans retinoic acid (ATRA) therapy to efficiently sensitize AML cells to undergo differentiation and decrease their leukemia-initiating capability in human xenograft models [115]. Consistently, the studies of Harris and colleagues also indicated the therapeutic potential of LSD1 inhibitors on AML, albeit the long-term effect of LSD1 inhibitors remains to be evaluated [116]. Fur-

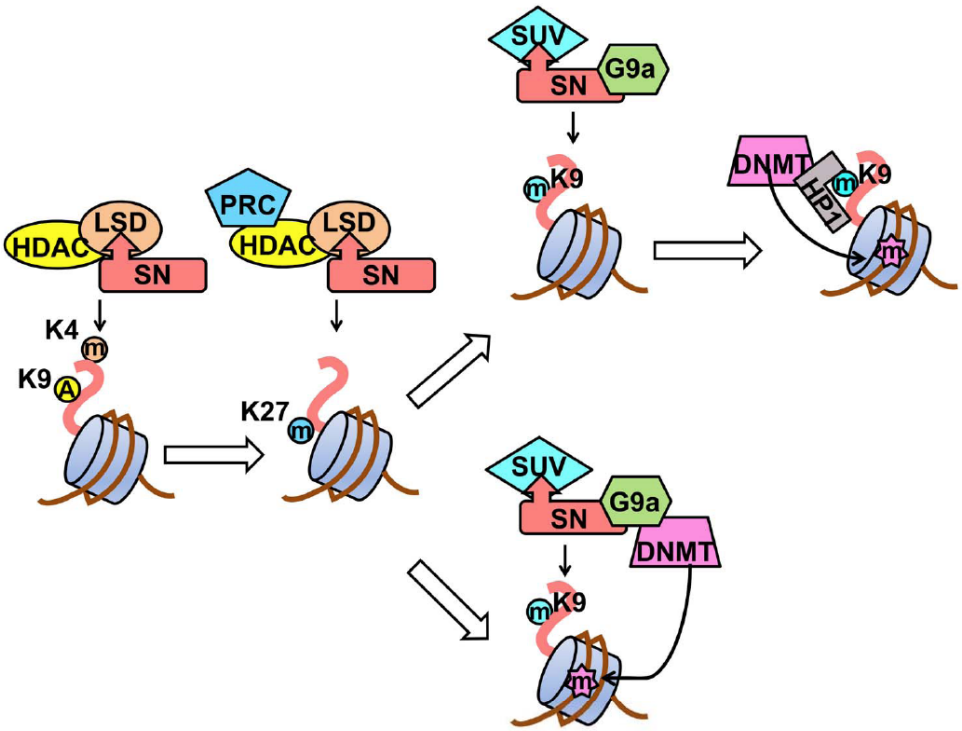

Fig. (1). Snail mediates epigenetic events in a hierarchic fashion to suppress E-cadherin. First, Snail uses its SNAG domain to recruit LSD1/HDAC complex for histone H3K4 demethylation and H3/H4 deacetylation. Histone deacetylation may facilitate the recruitment of PRC2 to initiate the transcription silencing process, while H3K4 demethylation favors the association of G9a and Suv39H1. G9a interacts with DNMTs, therefore recruiting them to E-cadherin promoter; alternatively, G9a and Suv39H1 create H3K9 methylation marks, which in turn recruit HP1 and DNMTs to the promoter area for gene silencing.

thermore, inhibitors targeting histone methyltransferases including G9a are under development and potential clinical trials would depend on the satisfactory results of those preclinical studies [117]. Overall, while clinical data of epigenetic drugs against cancerous EMT are still limited, growing evidence highlights the therapeutic potential of these inhibitors. An important issue during the development of EMT-targeting epigenetic drugs is to test whether those candidates can affect other "off-target" molecules, leading to undesirable side effects. The in silico off-target prediction technology based on the chemical structure of the compounds will be helpful during the drug design and test process. As the last and intriguing point of view, considering the hierarchic and interdependent nature of the EMT regulation program, SNAG-mimicking compounds may efficiently interrupt this epigenetic modulation cascade by competing with Snail for binding of histone-modifying enzymes such as LSD1, HDACs and Suv39H1, therefore inhibiting their recruitment by Snail to the E-cadherin promoter. In this regard, these compounds may work in great synergy with other treatment strategies for the cure of patients with EMT-related diseases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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